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Phosphorylation and Internalization of gp130 Occur After IL-6 Activation of Jak2 Kinase in Hepatocytes

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Recent evidence has shown that members of the Jak kinase family are activated after IL-6 binds to its receptor complex, leading to a tyrosine phosphorylation of gp130, the IL-6 signal-transducing subunit. The different members of the IL-6 cytokine subfamily induce distinct patterns of Jak-Tyk phosphorylation in different cell types. Using monospecific antibodies to gp130, Jak2 kinase, and phosphotyrosine, we investigated the kinetics of IL-6 stimulation of members of this pathway in primary hepatocytes. Our findings show that Jak 2 is maximally activated within 2 min of exposure to IL-6, followed by gp130 phosphorylation that reaches its peak in another 2 min then declines to basal level by 60 min. In vitro phosphorylation experiments show that activated Jak 2 is able to phosphorylate both native gp130 and a fusion peptide containing its cytoplasmic domain, demonstrating gp130 is a direct substrate of Jak 2 kinase. Experiments designed to explore the cell surface expression of gp130 show that ≥2 h are required to get a second round of phosphorylation after the addition of more cytokines. This finding suggests that activated gp130 is internalized from the cell surface after IL-6 stimulation. Additional experiments using protein synthesis inhibitors reveal that new protein synthesis is required to get a second cycle of gp130 phosphorylation indicating gp130 must be synthesized de novo and inserted into the membrane. These findings provide strong evidence that down regulation of the IL-6 signal in hepatocytes involves the internalization and cytosol degradation of gp130.

INTRODUCTION

The interleukin-6 (IL-6) signal transducing protein, gp130, has recently been shown to be a shared receptor subunit for at least four other cytokines, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor (CNTF), and IL-11 (Bazan, 1991; Gearing *et al.*, 1992; Ip *et al.*, 1992; Miyajima *et al.*, 1992; Stahl *et al.*, 1993). The members of the IL-6 cytokine subfamily that use gp130 are able to stimulate the expression of some of the same genes (Rose and Bruce, 1991; Baumann *et al.*, 1993). Through specific binding receptors and a shared signal-transducing subunit (gp130), both redundancy and pleiotrophy are functional characteristics of members of this cytokine family (Lord *et al.*, 1991; Kishimoto *et al.*, 1992; Miyajima *et al.*, 1992). There are instances, however, in which cellular responses to different cytokines of the family differ quantitatively, implying at least a "preferred" cytokine for a specific cell response (Thoma *et al.*, 1994). For example, IL-6 is a more potent inducer of fibrinogen expression than LIF or IL-11 even though the latter cytokines do upregulate fibrinogen production (Baumann and Schendel, 1991; Lord *et al.*, 1991). These observations suggest that specificity of responses likely resides in subtle differences of components that participate in the intracellular pathway.

It has been postulated for several years that the common signal transducing subunit gp130 should be a substrate for an intracellular kinase because it does not possess any intrinsic kinase activity itself (Hibi *et al.*, 1990; Saito *et al.*, 1992; Wang *et al.*, 1992). Thus an intensive search has been carried out to identify the intracellular kinases that phosphorylate gp130. Within the past year results have been reported in which the Jak kinase family, consisting of three members, Jak1, Jak2, and Tyk2 (Wilks, 1989; Firmbach-Kraft *et al.*, 1990; Wilks *et al.*, 1991), has been shown to be involved in the signaling process of IL-3 (Silvennoinen *et al.*, 1993), erythropoietin (Witthuhn *et al.*, 1993), interferon α, γ (Velazquez *et al.*, 1992; Muller *et al.*, 1993; Watling *et al.*, 1993), and growth hormone (Argetsinger *et al.*, 1993). It has been documented that different Jak kinases

are activated by each member of the IL-6 cytokine family (Lutticken *et al.*, 1994; Stahl *et al.*, 1994), and it also appears that the same cytokine activates a distinct pattern of Jak kinases in different cell types (Muller *et al.*, 1993; Watling *et al.*, 1993; Stahl *et al.*, 1994). The activation of Jak2 kinase has not been investigated in hepatocytes nor has the direct phosphorylation of gp130 by Jak kinases been shown. In present study we used specific antibodies to elucidate in detail the activation of Jak2 kinase and gp130 phosphorylation in primary hepatocytes. Additional experiments using endogenous or recombinant gp130 as a substrate in kinase assays demonstrate that gp130 molecule is directly phosphorylated by Jak2 kinase.

Furthermore, our studies reveal that the time course of gp130 phosphorylation disappearance is ~1 h. Experiments were performed to determine whether gp130 could be restimulated using a different cytokine (CNTF) and its cognate receptor (Davis *et al.*, 1991; Ip *et al.*, 1993). The gp130 molecule cannot be rephosphorylated by the addition of either IL-6 or CNTF, suggesting that gp130 has been removed from the cell surface and made unavailable for further activation. Experiments utilizing protein synthesis inhibitors (cycloheximide and puromycin) show that protein synthesis is required for a second round of IL-6 signaling, implying that the activated gp130 molecules are internalized and then degraded.

MATERIALS AND METHODS

Materials

Tissue culture media, antibiotics and fetal bovine serum were purchased from GIBCO BRL (Gaithersburg, MD) and Hyclone (Logan, UT), respectively. The glutathione-S-transferase (GST) fusion protein expression and purification system was from Pharmacia (Piscataway, NJ). Restriction and modification enzymes were from Promega (Madison, WI) and New England Biolab (Beverly, MA). Radionucleotides were supplied by Amersham (Arlington Heights, IL). All other chemicals were from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MO).

Cell Culture

Rat primary hepatocytes were isolated from adult Sprague Dawley strain (100–200 g) by a modification of the collagenase perfusion technique and plated on fibronectin-coated tissue culture plates as previously described (Nesbitt and Fuller, 1991). Cells (1×10^7) were plated on 100 mm tissue culture plates and cultured in William's E media (minus arginine) for 18–24 h before the stimulation of cells.

Expression and Purification of gp130 Fusion Protein

Two primers were designed according to the sequence of rat hepatic gp130 in the cytoplasmic region between residues 645 and 918 (Wang *et al.*, 1992). The forward primer 5′-TTGGATCCGGAGGCCTAATTAAAAAACACATC-3′ and reverse primer 5′-CCCTCTAGATCACTGTGGCATGTAGCC-3′ were used to amplify the complete cytoplasmic domain by polymerase chain reaction using the full length gp130 cDNA as a template. The amplified fragment was cloned into GST fusion protein expression vector pGEX-2T (Pharmacia). The designed 5′ primer contains two additional glycine codons at the boundary between the GST and gp130 fusion-protein. These additional glycine residues allow for more efficient thrombin cleavage.

For expression and purification of the gp130-GST fusion protein, the DH5α *Escherichia coli* strain transformed with pGEX-2T-gp130 was cultured overnight in LB medium containing 100 μg/ml ampicillin. The overnight culture was then diluted 10-fold with fresh LB medium and incubated for 60 min, followed by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside, after which the induction proceeded for 5 h. The bacteria were collected by centrifugation and resuspend in ice-cold phosphate-buffered saline (PBS), containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After sonication of 4×10 s, the cell lysate was adjusted to 1% Triton X-100 by adding 10% stock solution and then centrifuged ($10\,000 \times g$) for 10 min at 4°C to remove insoluble materials and intact cells The supernatant was applied to glutathione-sepharose affinity column (Pharmacia), and the bound fusion protein was eluted with 5 mM reduced glutathione in 50 mM tris(hydroxymethyl) aminomethane (Tris)-HCl, pH 8.0. To remove the GST portion from the fusion protein (GST-gp130), the sample was digested with 0.1% human thrombin and loaded onto a glutathione-sepharose column (Ausubel *et al.*, 1989). The flow-through fraction contained gp130 protein. The purified gp130 protein was either used to immunize rabbit or as a substrate in Jak2 kinase activity assay.

Cytokines and Antibodies

Recombinant murine IL-6 was expressed and purified as previously documented (Grenett *et al.*, 1991). Murine CNTF was a generous gift from Dr. Rick Scott (Cephalon, West Chester, PA). Polyclonal antibodies against gp130 were prepared by immunizing a rabbit with purified rat gp130 cytoplasmic peptide. Briefly, 200 μg of gp130 was mixed with equal volume of Freund's complete adjuvant and injected intradermally into 10–12 sites along the upper back of the rabbit. After 3 wk, 100 μg antigen and Freund's incomplete adjuvant mixture was injected subcutaneously into two to four additional sites. The antiserum titer was tested on day 31 by Western blot. The animal was boosted by an intramuscular injection of 100 μg of gp130 protein then rested for an additional week before bleeding. High affinity antibodies to gp130 cytoplasmic domain were partially purified by passing the serum over a protein A-sepharose resin (Ey *et al.*, 1978). The antisera against Jak1 and Jak2 have been described (Silvennoinen *et al.*, 1993) and were provided as a generous gift from Dr. James Ihle (St. Judes Research Hospital, Memphis, TN). Anti-phosphotyrosine antibody (4G10) was purchased from UBI (Lake Placid, NY).

Immunoprecipitation and Western Blot Analysis

Confluent cultures of rat primary hepatocytes (2×10^7 cells) were incubated 16 h before beginning any experiment. Thirty minutes before the addition of cytokines, fresh medium containing 50 mM sodium orthovanadate was added to the culture, then treated with cytokines for the indicated time and dose. After treatment with the cytokines, cells were washed with ice-cold PBS/50 mM Na_3VO_4 and subsequently resuspended in 700 μl of cell lysis buffer (10 mM Tris-HCl pH7.8, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM aprotinin, and 1 mM leupeptin) and incubated on ice for 30 min. The lysates were centrifuged at $10\,000 \times g$, 4°C, and precleared for 2 h with protein A-sepharose that was preincubated with normal rabbit serum. The precleared supernatant was then immunoprecipitated overnight at 4°C with specific antibodies and pelleted by protein A-sepharose. The immunoprecipitates were washed gently with lysis buffer six times and then boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The resolved proteins were detected by enhanced chemiluminescence (ECL) system (Amersham) with indicated primary antibodies and 1:30 000 dilution of anti-rabbit Ig or anti-mouse Ig-horseradish peroxidase (HRP) conjugate from Promega.

Stripping and Reprobing of Western Blot Membranes

To remove the primary and secondary antibodies from the filter for reprobing with a different antibody, the filter was incubated in stripping buffer (62.5 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol, 2% SDS) at 50°C for 30 min with occasional agitation. The filter was then washed with tris-buffered saline containing Tween-20 (TBS-T, 10 mM Tris pH 8.3, 0.15 M Na Cl, 0.1% Tween-20) twice for 10 min and blocked in 3% milk and 1% bovine serum albumin in TBS-T for 1 h, followed by immunoblot as described above.

In Vitro Kinase Assay and Phosphorylation of gp130 Protein

Immunoprecipitated complexes were washed twice with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) and then resuspended in 50 ml of kinase buffer (10 mM HEPES pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$). [γ-^{32}P] ATP was added to yield a final concentration of 1 mCi/ml (Amersham). After incubation for 10 min at 30°C, the reaction was stopped with the addition of 10 mM EDTA. The samples were boiled in SDS sample buffer, resolved by SDS-PAGE, and visualized by autoradiography. In the experiments of gp130 phosphorylation [γ-^{32}P] ATP was substituted by nonradioactive ATP, and immunoprecipitated native gp130 or purified recombinant gp130 protein was also added to kinase reaction as substrate. Then the tyrosine-phosphorylated proteins were detected by anti-pTyr blot using the ECL system.

RESULTS

Jak2 and gp130 Are Rapidly Tyrosine Phosphorylated by IL-6 Stimulation in Hepatocytes

Recent reports have shown that individual cytokines of the IL-6 family stimulate distinct patterns of Jak-Tyk phosphorylation in different cell lines (Stahl *et al.*, 1994). We therefore specifically examined Jak2 kinase activation by IL-6 in rat primary hepatocytes. Solubilized cellular proteins from IL-6–treated rat hepatocytes were immunoprecipitated using antibodies to gp130 or Jak2 and analyzed by anti-phosphotyrosine antibody (4G10) immunoblot. As shown in Figure 1, IL-6 stimulates gp130 phosphorylation in a time-dependent manner. The phosphorylation of gp130 was evident at 2 min, reached the maximum level at 5 min, then became undetectable at 60 min. The time course for Jak2 phosphorylation presented in Figure 2A showed that it attained a peak value between 2 and 5 min. When the same filter was stripped and reprobed with anti-Jak2 antibody, the recognition of the p130 band by this antibody confirmed that it was Jak2 protein and moreover that each lane had been loaded with an comparable amount of protein (Figure 2B). Comparison of the first appearance of phosphorylation of gp130 and Jak2 revealed a subtle but reproducible lag time between Jak2 and gp130, demonstrating phosphorylation of Jak2 kinase precedes that of gp130. Experiments using various concentration of IL-6 also showed that the phosphorylation of gp130 and Jak2 occurred at physiological concentration of IL-6 as low as 5.0 ng/ml (~24 pM).

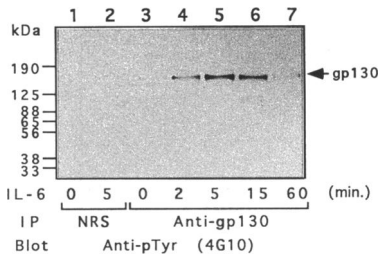

Figure 1. IL-6 stimulates gp130 tyrosyl phosphorylation. Primary hepatocytes were incubated with recombinant murine IL-6 (rmIL-6) for indicated time (0–60 min) at a concentration of 100 ng/ml. Cells were lysed and immunoprecipitated with anti-gp130 or normal rabbit serum (NRS). The precipitated proteins were subjected to 5–15% gradient SDS-PAGE for Western blot analysis using anti-phosphotyrosine (anti-pTyr) from UBI (4G10). The signal was developed by utilizing ECL.

IL-6 Activates Jak2 Kinase In Vitro

Tyrosine phosphorylation of proteins is commonly associated with the activation of kinase activity (Hanks *et al.*, 1988). However the Jak2 phosphorylation stimulated by IL-6 does not necessarily prove that IL-6 activates Jak2 kinase, because it is possible that another intermediate kinase may be activated by IL-6 and then phosphorylates Jak2 protein. To confirm that IL-6 activates Jak2 kinase directly, we examined the in vitro activity of Jak2 kinase in IL-6–treated primary hepatocytes. After cells were stimulated with IL-6 for the times indicated, cell lysates were prepared and immunoprecipitated using anti-Jak2 serum. The immunoprecipitated proteins were added to an in vitro kinase assay system in the presence of [γ-^{32}P] ATP. The ^{32}P-labeled proteins were resolved by SDS-PAGE, transferred to a nitrocellulose filter, and visualized by autoradiography (Figure 3A). Immunoprecipitates of extracts with anti-Jak2 serum from IL-6–stimulated cells showed strong kinase activity. In contrast, immunoprecipitates of extracts with normal rabbit serum had no detectable kinase activity. The maximum phosphorylation of Jak2 (or kinase activity) was observed in the cell extracts stimulated by IL-6 for 5 min. This dominant phosphoprotein was also detected when this same filter was blotted with 4G10 antibody, indicating the phosphorylation resided in tyrosine residues (Figure 3B). These data show that IL-6 stimulates tyrosine kinase activity of Jak2 that also induces autophosphorylation of Jak2.

Both Native and Recombinant gp130 Protein Are Tyrosine Phosphorylated by Activated Jak2 Kinase

Jak kinases are associated with gp130 in several cell types (Lutticken *et al.*, 1994; Stahl *et al.*, 1994). Data presented in this study also show that IL-6 activates Jak2 kinase (Figure 3). Furthermore, the appearance of gp130 phosphorylation corresponds in time with Jak2 activation suggesting that gp130 is a substrate for Jak2

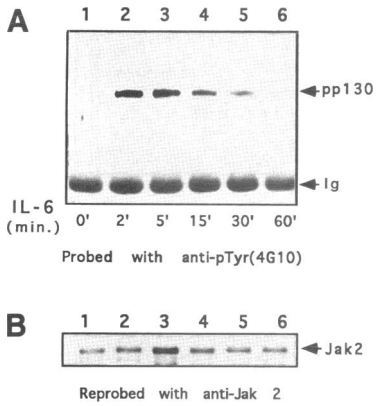

Figure 2. IL-6 induces Jak2 phosphorylation. Cells were stimulated as described in Figure 1. Cell lysates were immunoprecipitated with antibody to murine Jak2 kinase and resolved on SDS-PAGE. Phosphorylated Jak2 protein was detected by Western blot analysis (A) with the first antibody 4G10 and the secondary antibody to anti-mouse Ig conjugated with HRP. The same filter was stripped and reprobed with anti-Jak2 and anti-rabbit Ig HRP conjugate (B), showing that a similar amount of Jak2 protein was loaded in each lane.

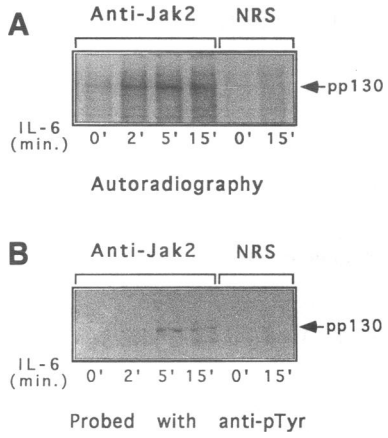

Figure 3. IL-6 activates Jak2 kinase in vitro kinase assay. Cell lysates from rmIL-6 (100 ng/ml)–stimulated primary hepatocytes for various times (0–15 min) were immunoprecipitated with anti-Jak2 or NRS. The precipitated proteins were used for in vitro kinase assay in the presence of [γ-^{32}P] ATP. The products of the reaction were resolved by SDS-PAGE, transferred to nitrocellulose, and detected by autoradiography (A). The filter was subsequently probed with anti-phosphotyrosine (anti-pTyr) to show that phosphorylation occurs on tyrosine residues (B).

kinase. To demonstrate this more directly, experiments were carried out that used the immunoprecipitated endogenous gp130 from unstimulated cells as the substrate. Jak2 that had been isolated from IL-6–stimulated cells and the gp130 from unstimulated cells were mixed in conditions for in vitro phosphorylation. The products of this reaction were analyzed by separating them on SDS-PAGE and transferring to a nitrocellulose membrane for anti-phosphotyrosine blot (Figure 4A). The findings revealed that the endogenous gp130 proteins from unstimulated cells were phosphorylated by the IL-6–activated Jak2 kinase. The tyrosine-phosphorylated gp130 proteins were only detected in the extracts of IL-6–stimulated cells that immunoprecipitated with anti-Jak2, not in normal rabbit serum immunoprecipitation of either stimulated or unstimulated cells (Figure 4A, lanes 6 and 7).

To demonstrate convincingly that Jak2 kinase, not a contaminating tyrosine kinase in the anti-Jak2 immunoprecipitate, directly phosphorylates gp130, we performed the following experiment. Both unstimulated and IL-6–treated cells were immunoprecipitated with anti-Jak2 and preimmune serum under conditions identical to those employed above, and a anti-phosphotyrosine antibody (4G10) was used to detect tyrosine kinases present in the immunoprecipitate. As shown in Figure 5A, only one tyrosine-phosphorylated band, which migrated to 130 kDa position, was visualized. When the same filter was reprobed with anti-Jak2, the identity of Jak2 kinase was confirmed. Moreover, gp130 was not co-immunoprecipitated with anti-Jak2 nor Jak2 with anti-gp130 in the NP-40 lysis buffer we used (Figure 6B). This is consistent with the studies carried out by others (Lutticken *et al.*, 1994; Stahl *et al.*, 1994), showing the protein association between gp130 and Jak kinase could only be detected using Brij 96 lysis buffer. The anti-Jak2 antibody recognized four bands in the immunoprecipitate, however, only the 130-kDa protein is the functional form (Silvennoinen *et al.*, 1993). These results indicate that the immunoprecipitation with anti-Jak2 does not contain either other tyrosine kinases or

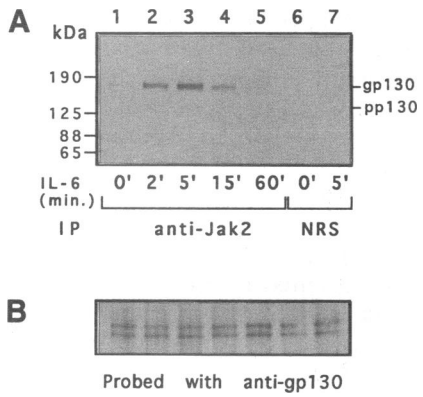

Figure 4. IL-6–activated Jak2 kinase phosphorylates endogenous gp130 protein from unstimulated cells. The cell lysates for IL-6–stimulated cells were precipitated with anti-Jak2 or NRS. These immunoprecipitates were incubated with endogenous gp130 proteins (from 2×10^7 cells) that had been precipitated from unstimulated cells with a specific antibody to the cytoplasmic domain of rat gp130 molecules. The in vitro kinase assay was performed as described in MATERIALS AND METHODS. The phosphorylated proteins were applied to SDS-PAGE and analyzed by anti-phosphotyrosine blot (A). The same filter was reprobed with anti-gp130 as shown in B. The antibody to gp130 always recognizes two forms of gp130 in hepatocytes.

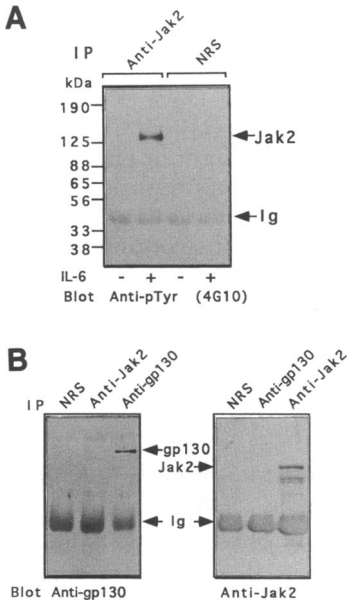

Figure 5. The antibody to Jak2 specifically immunoprecipitates Jak2 kinase. Primary hepatocytes were stimulated with rmIL-6 (100 ng/ml) for 5 min. Cells were lysed and immunoprecipitated with anti-Jak2 or NRS. The precipitated proteins were subjected to 5–15% gradient SDS-PAGE for Western blot analysis using anti-phosphotyrosine (4G10). The autophosphorylated tyrosine kinase was detected by utilizing the ECL system (A). In B, cell lysates from IL-6–stimulated cells were immunoprecipitated with the indicated antibodies or NRS and subsequently blotted with anti-gp130 or anti-Jak2, respectively.

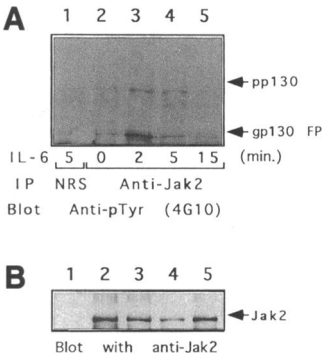

Figure 6. The cytoplasmic domain of recombinant gp130 is phosphorylated by activated Jak2 kinase. The experiment was carried out similarly to that shown in Figure 4 except purified gp130 cytoplasmic domain was used as a substrate (1 μg per reaction). The anti-phosphotyrosine blot result is shown in A. The phosphorylated gp130 fusion protein (gp130 FP) and activated Jak2 (pp130) are indicated by two arrows. The same filter was reprobed with anti-Jak2 (B) indicating that an approximate equal amount of immunoprecipitated Jak2 kinase was used in every reaction except NRS control.

endogenous gp130. As an extension of these findings, a fusion protein containing the cytoplasmic domain of gp130 was used as the substrate for the activated Jak2 kinase. The results shown in Figure 6A indicate that the gp130 fusion protein could also serve as a substrate for the Jak2 kinase. The results of these two experiments provide direct experimental data that gp130 is a specific substrate of Jak 2 and further demonstrate that IL-6 stimulates Jak 2 kinase in primary hepatocytes. It should be noted that IL-6 also activated Jak1 kinase and then caused the phosphorylation of gp130 in primary hepatocytes (unpublished results). This latter observation implies that both kinases are stimulated by IL-6 in the hepatocyte and likely cooperate in the phosphorylation of gp130.

Genistein Inhibits Jak 2 Kinase Activity and Phosphorylation of gp130

If Jak2 kinase phosphorylates gp130, the inhibition of Jak2 kinase activity should also cause a decrease of gp130 phosphorylation. We utilized a highly specific tyrosine kinase inhibitor, genistein (Akiyama *et al.*, 1987), to explore further the Jak2 kinase activity and gp130 phosphorylation. The primary hepatocytes were incubated with fresh media in the absence or presence of genistein for 30 min. Then cells were stimulated by IL-6 (100 ng/ml) for 5 min, and the extracts of cell lysates were applied to immunoprecipitation with indicated antibodies (Jak2, gp130, and normal rabbit serum control). The phosphorylated proteins were then detected by anti-phosphotyrosine immunoblot (Figure 7). It was shown that genistein blocked the phosphorylation of Jak2 kinase and subsequently caused a significant decrease of gp130 phosphorylation (lanes 9 and 6, respectively). The phosphorylated protein was not present in unstimulated cells or immunoprecipitation with normal rabbit serum. It is interesting to point out that the phosphorylated gp130 migrated to ~150 kDa and the phosphorylated Jak2 migrated to 130 kDa position in agreement with a previous study (Stahl *et al.*, 1994). The experimental results here support the idea that Jak2 kinase directly phosphorylates gp130 protein in IL-6 signaling pathway in hepatocytes.

The gp130 Is Internalized After IL-6 Stimulation

A common mechanism that cells employ to modulate a stimulatory signal is to endocytose the ligand-receptor complex thereby making receptors unavailable for signaling (Carpenter and Cohen, 1976; Mellman *et al.*, 1986; Tran *et al.*, 1987). The fate of the receptor in such a complex can follow one of several possibilities. For example, once the ligand-receptor complex is internalized, the ligand can dissociate from the receptor and then the receptor can return to the cell surface, whereas

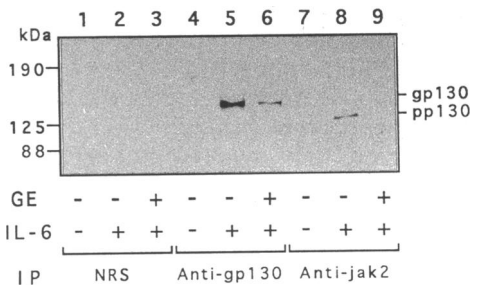

Figure 7. Genistein inhibits Jak2 kinase activity and phosphorylation of gp130. Primary hepatocytes were incubated with fresh media in the absence (−) or presence (+) of genistein (GE) for 30 min then stimulated with rmIL-6 (100 ng/ml) for 5 min. Cell lysates from these treated or untreated cells were immunoprecipitated with antibody to gp130, Jak2, or NRS control. Western blot was performed using anti-pTyr (4G10) antibody. Phosphorylated gp130 and Jak2 (pp130) are represented by two small bars.

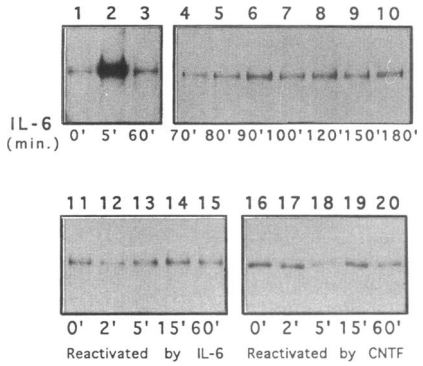

Figure 8. Reactivation of gp130 by IL-6 and CNTF. In the top part (lanes 1–10), cells were stimulated by rmIL-6 (100 ng/ml) for the indicated times. In the bottom panel, cells were first stimulated with IL-6 for 1 h, followed by addition of fresh cytokine (IL-6, lanes 11–15; or CNTF, lanes 16–20) for different time periods. Cells were immediately lysed for immunoprecipitation using anti-gp130. The phosphorylated gp130 was detected by 4G10 immunoblot.

the ligand is processed to the lysosome for degradation (Goldstein *et al.*, 1985; Marshall, 1985). Another possible route for the receptor to follow is after internalization both the ligand and receptor remain together and are processed to the lysosome for degradation (Brown and Greene, 1991; Sorkin and Waters, 1993; Straight *et al.*, 1993). We have previously reported that 125-iodine–labeled IL-6 is rapidly internalized and degraded (Nesbitt and Fuller, 1992b), however, in those studies we were unable to determine if this internalization also included the gp130 subunit.

Experiments were carried out here to determine the time course of activation and reactivation of gp130 (Figure 8). The time course of gp130 phosphorylation and disappearance has been shown to occur within 1 h (see lanes 1–10). To ascertain if a second round of signaling could occur at the end of the first signaling period (60 min), we added fresh IL-6 to the cells and then examined for gp130 phosphorylation. The results shown in the lower part of Figure 8 (lanes 11–15) demonstrate that the addition of more IL-6 had no effect in initiating gp130 phosphorylation. The possibility exists that the IL-6 cognate receptor (gp80) could be a limiting factor so that reactivation of gp130 is not possible. Because CNTF binds to its own receptor before association with gp130 (Ip *et al.*, 1992) and stimulates an acute phase response in liver cells, we used this cytokine to determine if a second gp130 phosphorylation could be generated after an initial IL-6 activation. As shown in Figure 8 (lanes 16–20), CNTF also failed to generate a second wave of gp130 phosphorylation indicating that gp130 was the limiting subunit in this reactivation process.

To determine the length of time that the cell required to be able to respond to a second IL-6 signal, we pulsed the cells with IL-6 after the initial signaling event had occurred. Cells were first treated with IL-6 for 1 h, then fresh media containing no cytokine was added. At the end of 1, 2, and 4 h, the cells were restimulated by adding IL-6 for 5 min before processing for gp130 phosphorylation (Figure 9). The results show that a small amount of gp130 activation could be detected at 1 h but had returned to nearly full responsiveness by 4 h. On the other hand, the gp130 phosphorylation signal in the continual presence of IL-6 was somewhat higher than background but no pronounced phosphorylation was detected (Figure 8, lanes 3–10). These findings together predict that the gp130 molecule is removed from cell surface and internalized with the bound ligand in the IL-6 signal process.

Protein Synthesis Is Required for gp130 Reentry to Signal Pathway

Two possible explanations could account for the fate of internalized gp130 molecules. First, it is possible that activated gp130 is internalized and, following separation from the ligand, could recycle to the cell surface. Second, gp130 could be targeted for degradation, and new gp130 has to be synthesized before it appears in the membrane. To determine if protein synthesis is required for the gp130 reentry to the signaling pathway, we used two well-defined protein synthesis inhibitors cycloheximide (CHX) and puromycin (PUR). It is conceivable that transcription may be essential for the cell to reset the gp130 signal, therefore we also used the transcriptional inhibitor, actinomycin-D (ACT-D). Hepatocytes were stimulated with IL-6 for 1 h to remove the cell surface protein of gp130, after which fresh media was added in the presence of indicated inhibitors and incubated for an additional 4 h. Cells were then restimulated by adding IL-6. Phosphorylation of gp130 was observed both in noninhibitor-treated cells and cells treated with actinomycin D. In contrast, gp130 phosphorylation could not be detected in cells treated with either of the protein synthesis inhibitors (Figure 10A). As a control, cells were treated with CHX, PUR, and ACT-D for 4 h then stimulated with IL-6. These inhibitors had no effect on the capacity of IL-6 to activate gp130 molecules that had already been expressed on the cell surface (Figure 10B). The disappearance of gp130 initiated by IL-6

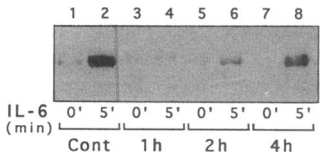

Figure 9. Accumulation and activation of cell surface gp130. Cells were stimulated with rmIL-6 (100 ng/ml) for 1 h, then changed to fresh media containing no cytokine, and restimulated with IL-6 for 5 min at different times (1, 2, and 4 h after initial stimulation). Lanes 1 and 2 serve as a positive control in which cells received IL-6 only once. Cell lysates were immunoprecipitated with anti-gp130 and blotted with the 4G10 monoclonal antibody against phosphotyrosine.

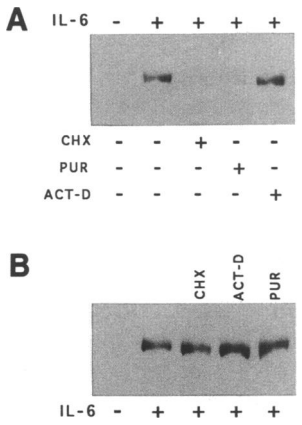

Figure 10. Protein synthesis is required for new gp130 secreting into the cell membrane. (A) Primary hepatocytes were treated with rmIL-6 (100 ng/ml) for 1 h and incubated with fresh media containing no cytokine in the presence (+) of indicated protein synthesis inhibitors, CHX (2.5 μg/ml), PUR (2.5 μg/ml), and ACT-D (2.5 μg/ml) for 4 h. Cells were restimulated with rmIL-6 again for 5 min. After immunoprecipitation with anti-gp130, the phosphorylated proteins were detected by anti-phosphotyrosine blot (4G10). (B) Cells were incubated with different inhibitors for 4 h then stimulated by rmIL-6 (100 ng/ml) for 5 min to test the effects of these inhibitors on the phosphorylation of gp130 stimulated by IL-6. Phosphorylated gp130 were immunoprecipitated and detected as in A.

binding was also detected directly by anti-gp130 blot. Cells were treated as previously described and lysed for immunoprecipitation and blot with a monospecific antibody to gp130 protein (Figure 11A). The diminution of gp130 was detected in the presence of protein synthesis inhibitors (CHX and PUR) that prevented the reappearance of newly synthesized gp130. These inhibitors had not effect on gp130 detection if the cells were not stimulated by IL-6 (Figure 11B). Taken together, these findings provide evidence that gp130 is internalized and degraded after IL-6 stimulation and that new gp130 must be synthesized, processed, and inserted into the membrane before additional signals can be generated.

DISCUSSION

IL-6 is the major signaling cytokine for the liver during an acute inflammatory response. The currently accepted paradigm for this signal process is that IL-6 first binds to a cognate receptor (gp80) at a relatively low affinity, and then this complex associates with a second transmembrane subunit (gp130) forming a high-affinity ligand receptor complex (Taga *et al.*, 1989; Hibi *et al.*, 1990). It is now believed that the first event in second phase of this association is to initiate dimerization of the gp130 subunits. The dimer is then stabilized through an interchain disulfide linkage between two gp130 molecules (Murakami *et al.*, 1993). The third event that has been measured is the phosphorylation of gp130 resulting in a conformational change that is involved in relaying the IL-6 signal (Murakami *et al.*, 1991). Because gp130 itself is not a kinase, the last event must include the activation of yet another molecule. Recently it has been shown that a family of cytoplasmic kinases known as the Jak kinase family is involved in the IL-6 signal pathway (Lutticken *et al.*, 1994; Stahl *et al.*, 1994).

gp130 Is Directly Phosphorylated by IL-6-activated Jak Kinase

In this study we examined in more detail the early events of the IL-6 signaling pathway in primary hepatocytes. In particular, we wished to document the involvement of the Jak kinases in the activation processes in untransformed cells. To this end we produced a monospecific polyclonal antibody to the cytoplasmic domain of gp130 to identify the presence of gp130 irrespective of whether or not it had been phosphorylated. Using this antibody probe has enabled us to explore the interaction of the Jak kinases that phosphorylate gp130 in hepatocytes. Although published results indicate close association of gp130 and Jak family kinases (Lutticken *et al.*, 1994; Stahl *et al.*, 1994), the direct linkage between them is not completely understood. It is important to understand the step-by-step details of this pathway. Therefore, we examined the time course of Jak2 and gp130 activation and demonstrated that there is a subtle but detectable difference in the appearance of phosphorylation of each of the two proteins, suggesting that the Jak2 kinase may be "activated" first by ligand-induced homodimerization of gp130 and become autophosphorylated. Our finding that Jak2 kinase is phosphorylated and activated by IL-6 stimulation in primary hepatocytes also extended findings of the previous study that Jak1 and Tyk2 kinases are activated in HepG2 cells (Lutticken *et al.*, 1994). Because Jak family kinases are intimately associated with the cytoplasmic domain of gp130 constitutively but only activated by ligand binding (Stahl *et al.*, 1994), the process of dimerization of gp130 is likely the critical physical rearrangement that stimulates Jak2 phosphorylation by as yet an unknown mechanism.

We also reported here that either endogenous gp130 from unstimulated cells or purified recombinant gp130 fusion protein were directly phosphorylated by IL-6-activated Jak2 kinase. When Jak2 kinase activity was blocked by a tyrosine kinase inhibitor (genistein), the phosphorylation of gp130 protein was also inhibited. These data together indicated that gp130 is the first identified substrate for the Jak kinase family. Other potential candidate substrates for Jak kinases may include transcription factors, p91, a member of the interferon-

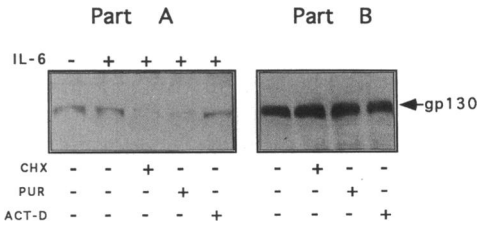

Figure 11. Detection of gp130 degradation after IL-6 stimulation. Cells were treated as described in Figure 10. After anti-gp130 immunoprecipitation, the pellets were fractionated by SDS-PAGE and blotted with an antibody to rat hepatic gp130.

α and -γ signaling pathway (Fu *et al.*, 1990; Kessler *et al.*, 1990; Decker *et al.*, 1991; Schindler *et al.*, 1992; Shuai *et al.*, 1992), and the acute phase response factor protein (Wegenka *et al.*, 1993). It is important to point out that both Jak1 and Tyk2 kinases are activated by IL-6 family cytokines in hepatoma cell line (Lutticken *et al.*, 1994). Additional experiments carried out in our laboratory showed that IL-6 activates Jak1 kinase and as effectively phosphorylates gp130 as Jak2 in primary hepatocytes. This latter observation implies that both kinases are stimulated by IL-6 in the hepatocyte and likely cooperate in the phosphorylation of gp130. The function of phosphorylated tyrosine residues of gp130 may serve as different binding sites to recruit various cellular proteins for potential substrates of Jak kinases then leading to a variety of downstream signal cascades.

Evidence for the Internalization of gp130 After IL-6 Stimulation

Although gp130 serves as the signal transducing protein for a number of cytokines, almost no information is available concerning how the gp130 signal is downregulated. Because gp130 is involved in sending a "generic signal" into the cell (in light of its pleiotrophy), several possibilities could be employed to shut off the signal and reset it for another signaling round. For example, a tyrosine phosphatase could remove the phosphate group of activated gp130 and allow resetting to occur while gp130 remains in the membrane, or the gp130-ligand complex could be internalized then dissociate from the complex and gp130 return to the membrane. A well recognized mechanism that many growth factors and cytokines employ is to downregulate their signals by internalization of the ligand-receptor complex. The internalized complex is routed to the lysosome where degradation occurs with or without receptor (Fujii *et al.*, 1986; Quarnstrom *et al.*, 1988; Galizzi *et al.*, 1989; Sorkin *et al.*, 1991; Sorkin and Waters, 1993). Some hint that this pathway may be the likely mechanism for gp130 was shown when we reported that IL-6 was internalized and degraded (Nesbitt and Fuller, 1992b).

The first clue to the possibility that the downregulation pathway used by gp130 involved internalization came from the reactivation experiments. After the initial gp130 phosphorylation occurred after IL-6 exposure for 1 h, which it is enough to endocytose 90% of bound ligand from the cell surface (Nesbitt and Fuller, 1992b), the addition of more IL-6 did not produce a second round phosphorylation of gp130. Because CNTF also uses gp130 as a signal transducing subunit and its own specific receptor for ligand binding, we added CNTF to the already IL-6–stimulated cells to determine the availability of surface expressed gp130. The finding that no additional phosphorylation occurred indicated that gp130 was not available for activation. To determine the length of time required to get a second phosphorylation event, experiments were performed in which the cells were "rested", i.e., IL-6 was removed and then at different times IL-6 again added to the cells. Phosphorylation could be detected after 1 h but was nearly fully restored by 4 h. On the other hand cells that were in the presence of IL-6 continuously after the initial signal did not give a strong second response at the end of 2 h post stimulation. We do note, however, a higher base-line of phosphorylation suggesting that when gp130 was again made available it was quickly phosphorylated and removed from the activation cycle. Taken together these data indicated that phosphorylated gp130 was removed from cell surface and internalized. Additional experiments were carried out using protein synthesis inhibitors as a way to determine if gp130 could return to the signaling cycle by a mechanism that did not involve new gp130 protein synthesis. The results of these experiments showed that in the presence of protein synthesis inhibitors (cycloheximide and puromycin) no IL-6 signal occurred after the initial signal was made. Unlike the IL-4 receptor in which cell surface reexpression is prevented by both cycloheximide and actinomycin-D (Galizzi *et al.*, 1989), gp130 reexpression is not affected by actinomycin-D within the time period examined (4 h). This finding is not unexpected in the view of the abundant mRNA level and long half life (12 h) of gp130 (Nesbitt and Fuller, 1992a; Saito *et al.*, 1992; Wang *et al.*, 1992). Our results clearly indicate that gp130 is likely degraded together with IL-6 and that the downregulation pathway is similar to that of other growth factors involving destruction of both the ligand and receptor.

In summary, the results of these experiments provide more detailed information about the IL-6 signal pathway in hepatocytes. Our data demonstrate clearly that the Jak kinases phosphorylate gp130 in this cell. Additionally, we provide the first information on the fate of gp130 after its activation by showing that it is internalized and most likely degraded.

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